(19) Japan Patent Office
(12) Japanese Unexamined Patent Application Publication

(11) Japanese Unexamined Patent Application Publication Number
S61-132189

(51) Int. Cl.[4]	Identification Symbol	JPO File Number	(43) Publication Date: June 19, 1986
C 12 N 15/00		7115–4B	
C 07 H 21/02		6742–4C	Number of inventions 2
//C 12 P 21/02		7235–4B	Request for examination Yes

(Total Pages: 7)

(54) Title of the Invention MESSENGER RNA OF SOYBEAN-STORED PROTEIN AND ITS METHOD OF PREPARATION

(21) Japanese Patent Application S59-254217
(22) Application Date: December 3, 1984

(72) Inventor Chikafusa FUKASAWA 2-802-203 Azuma, Sakura-mura, Nīhari-gun, Ibaraki-ken
(71) Applicant Director of Laboratory
General Foods Research, Ministry of Agriculture, Forestry and Fisheries
(74) Representative Fujirō KUBOTA, Attorney

SPECIFICATION

1. Title of the Invention
Messenger RNA of soybean-stored protein and its method of preparation
2. Scope of Patent Claims
(1) Messenger RNA of soybean-stored protein, attained from soybean seeds, with a fairly heavy fraction from 18S through fractionation using sucrose density slope centrifugation.
(2) Method of preparation for the messenger RNA of soybean-stored protein with the characteristics of extracting and fractionating messenger RNA from soybean seeds, attained with a fairly heavy fraction from 18S through sucrose density slope centrifugation.
3. Detailed Description of the Invention
This invention concerns messenger RNA (abbreviated as mRNA from hereon in) attained with a fairly heavy fraction from 18S through sucrose density slope centrifugation attained from soybean seeds, and its method of preparation, for soybean-stored protein.

Protein, fat, and dietary phytates are known to be main properties stored in soybean seeds, but it is necessary from first a seed physiology standpoint and food science perspective to know how these stored properties are synthesized and accumulated in the seed's ripening process.

The inventor of this invention has overlapped research on physiological roles and production methods of soybean-stored protein, and was successful at extracting mRNA that handles soybean-stored protein from soybean seeds in this process, and was successful at adjusting complementary DNA (abbreviated as cDNA from hereon in) from this mRNA. By manifesting DNA attained in this manner either in microorganism cells or in plant cells, the target soybean-stored protein can be manufactured.

This invention provides mRNA attained from soybean seeds with a fairly heavy fraction from 18S through sucrose density slope centrifugation fractionation, and its manufacturing methods, for soybean-stored protein.

The mRNA of this invention is attained with a fairly high fraction from 18S through agarose electrophoresis alongside fractionation using sucrose density slope centrifugation or gel filtration methods for soybean-stored protein as mentioned above, and can be manufactured by extracting and separating from soybean seeds.

Soybean seeds in various types of processes, such as soybean seeds in their ripening period, may be used as mRNA materials used for this invention.

A standard method is recommended to extract mRNA for soybean-stored protein from soybean seeds, regardless of the type of seeds. For example, combine the group with 2–5 containers of interface activators such as NP-40, SDS, Triton X-100 and phenol solution, and crush the cells using a physical method such as with a homogenizer or by using freeze thawing, solubilize, and add cold ethanol to the supernatant after centrifugation to sediment the RNA.

Also, when necessary, it is possible to extract the mRNA by sedimenting the polysomes partway through the soybean-stored protein synthesis process using antibodies that correspond to soybean-stored protein, and extracting using interface activators.

Also, purification of this invention's mRNA can be done by purification methods using adsorption columns such as oligo *d*T-cellulose or polyU-sepharose, or through fractionation through isokinetic sucrose density slope centrifugation methods. Using this kind of purification operation, the mRNA for this invention can be attained with a fairly heavy fraction from 18S.

To confirm that the mRNA attained as described above corresponds to soybean-stored protein, translate the mRNA to protein, and use a method that identifies protein, etc. using those antibodies. For example, reticulocyte-lyzate and wheat germ are a common non-cellular type frequently used when mRNA is translated into protein.

Thus, the maximum usage of the attained soybean-stored protein mRNA is to combine this mRNA in vitro with cDNA and insert the combination to an appropriate vector, etc., so that soybean-stored protein can be produced in a microorganism or plant, etc.

This type of cDNA combination can normally be done using the following methods within a test tube. With mRNA as the cast and oligo dT as the primer, the mRNA combines with the complementary single chain cDNA through reverse transcriptase under the existence of dATP, dGTP, dCTP, dTTP, and after the cast mRNA is degraded and removed through the alkaline process, oligo dC is added, then the double stranded cDNA is combined using either a reverse transcriptase with the single chain cDNA as the cast and oligo dG as the primer, or a DNA polymerase. Both DNA ends attained in this fashion can be processed with exonuclease when needed, and connect the appropriate DNA to each, and layer several bases for which annealing is possible. After some time, this mixture is inserted into a microorganism vector. To insert, cut the vector with an appropriate limited enzyme, and layer several bases in a combination so that linkers or annealing is possible. The double stranded DNA processed in this fashion mixes with the vector DNA, and is connected using ligase.

The attained recombinant DNA is inserted into the vector's host microorganism. Microorganisms of the escherichia genus such as escherichia coli, etc., microorganisms of the bacillus genus such as bacillus subtilis, etc., and microorganisms of the saccharomyces genus such as the saccharomyces cerevisiae, etc., are ideal host microorganisms. The vectors used in these microorganisms are indicated below. (See Protein Nucleic Acid Enzyme Volume 26, Number 4 (1981)) pSC101, pRK353, pRK646, pRK248, pDF41, etc. of EK-type plasmid vectors (stringent type); CalE1, pVH51, pAC105, RsF2124, pCR1, pMB9, BR313, pBR322, pBR324, pBR325, pBR327, pBR328, pKY2289, pKY2700, pKN80, pKC7, pKB158, pMK2004, pACYC1, pACYC184,

λdul, etc. of EK-type plasmid vectors (relaxed type); λgt·λc, λgt·λB, λWES·λB′, λZJvir·λB′, λALO·λB, λWES·Ts622, λDam, etc. of λgt-type phage vectors; charon 4A, charon 3A, charon 16A, charon 13A, charon 14A, charon 15A, charon 8, charon 10, charon 17, charon 20, etc. of charon vectors; L512, λZEQS, λZYV5ϕ, λZUVϕ2, λZUVϕ3, λYEQSϕ1, λYEQSϕ, λYEQSϕ3, λBam, λSst, etc. of tiollais group vectors; pTA1015, pLS15, pTA1020, pLS28, pLS13, pTA1050, pTA1060, pTA1030, pTA1031, etc. of bacillus subtilis plasmid vectors; pT127, pC194, pC221, pC223, pUB112, pUB110, pSA0501, pSA2100, pE194, pTP4, pTP5, etc. of plasmid vectors of staphylococcus origin; and yeast vectors pJDB219, YEp13, YRp5, YIp1, pYC, pTC2.

The microorganism vector, such as a Pstl or EcoR1 of pBR322, can manifest into soybean-stored protein by transforming into an appropriate host when it is inserted at a point that corresponds to the procedure's objectives.

The attained mRNA can be confirmed to have genetic information that corresponds to soybean-stored protein using the following method.

Identify that it is soybean-stored protein using the positive hybrid selection and in vitro translation method, using a type of reticulocyte lyzate or wheat germ.

Also, for plants, insert the gene with the pAL1050 vector including a T-DNA tmr region into the plant.

If inserting the recombinant DNA, connect the tmr leader arrangement to the in-frame, and use tmr for the termination codon region as well.

The aforementioned mRNA preparation method of soybean seeds in this invention shall be explained in detail through the usage example below. Further, the stored protein mRNA attained through the usage example below also can apply in the exact same manner as this invention,

and is included in the scope of this invention's specifications.

Embodiment 1

(1) Purify glycinin (one type of main soybean-stored protein) from a fully ripe soybean, separate acidity (abbreviated as A from hereon in) subunits, and purify. The molecular weight for these A subunits is approximately 35–40K, and shows strong chiasmal qualities that are strong in immunochemistry. Here, each A subunit is adjusted for specific antiserum. A preparation method for antiserum can be applied, for example, a method that repeats the absorbing operation by adding other lyophilizated acidity subunit protein powder to antiserum attained by thoroughly hyperimmunizing the specific subunits in rabbits. Specificity was tested according to the Ouchterlony's double gel diffusion method and Western Plot method.

(2) Prepare membrane-based polysomes from soybean cotyledons midway through their ripening period (38 days after flowering), and purify the mRNA using the SDS-phenol method and poly U-sepharose column method. Meanwhile, prepare all mRNA from cotyledons of this same period in a similar fashion. Fractionate a part of this total of mRNA samples using the sucrose density slope centrifugation method (sucrose 10% (w/w)–30% (w/w)), and identify the glycinin mRNA concentration fraction through immunochemistry analysis of the translated substance for red hemocyte acellular protein combination types.

(3) Create a cDNA that corresponds to all mRNA mentioned in above item (2) using the method described below.

i) In the alkali-degraded siliconized Eppendorf tube (1.5 ml) with the ss-c DNA combination and cast mRNA, add 10 μl of X10c DNA buffer (0.5 M-tris-hydrochloric acid, 1.4 M-chloride-potassium, 0.8 M-acetate-magnesium), 3 μl of RNasin (Seikagaku Corporation 40 u/μl), 5 μl of dATP, 5 μl of dGTP, 4 μl of dCTP and dTTP, 24 μl of oligo $(dT)_{12-18}$ (P-L Biochemicals Corporation 0.2 mg/ml), 8 μl of actinomycin-D (0.4 μg/μl), 1 μl of 0.1 MDTT, 6 μl of [α-$^{3\%}$p] dATP and 10 μl of mRNA (1 μg/μl, quenched after a 10 minute treatment under 65 °C)

in the order listed above, mix, centrifugate for one second, and gather the liquid at the bottom.

After incubating for 2 minutes at 42 °C, add 20 µl of AMV reverse transcriptase and mix gently, centrifugate for 1 second and incubate this mixture (100 µl) at 42 °C for 60 minutes.

Add 20 µl of 5M-chloride-sodium, 16 µl of 250 mMEDTA, 2 µl of 20% SDS and 62 µl of distilled water to the reactive liquid to stop the reaction.

Add 200 µl of phenol mixed solution (phenol solution saturated with 10 mMtris-hydrochloride (pH 8.3)–2mMEDTA) and shake vigorously.

Centrifugate and remove the aqueous layer, and add 122 µl of distilled water and 48 µl of 5M chloride-sodium solution to the remaining phenol layer and extract once again. After combining with the previous aqueous layer and carrying out ether processing so that the mixed phenol is removed, add 30 µl of acetate potassium (pH 5.0) and 600 µl of cold ethanol and precipitate the ethanol (30 minutes in dry ice and ethanol, or 1 hour at –70 °C).

3–5 µg of cDNA can be attained through this operation. This ss-cDNA is vacuum-dried, and dissolved by adding 45 µl of distilled water.

By adding and mixing 5 µl of 5N hydroxide sodium solution to this mixture, centrifugating for 1 second, gathering the liquid at the bottom of the tube, and incubating it overnight at 25 °C, the mRNA is degraded.

After adding 50 µl of Hepes-KOH (pH 7.4) buffer, place the mixture on an Ultrogel AcA44 column (gel bed height 28 cm) and fractionate approximately 0.6 ml at a time. Collect the void volume fraction (under these conditions, with a fraction number 6–8, measure using the Cerenkov method by checking with a GM survey meter or a liquid scintillation counter).

Add 1/10 the amount of this fraction of 3M acetate potassium and twice the amount of cold ethanol, spin down the mixture for an hour at –70 °C, and rinse the precipitated substance twice with 70% ethanol (superposition the ethanol gently so as not to peel off the precipitated substance, and rinse while centrifugating for 10 minutes), and vacuum-dry.

ii) Addition of dC-homopolymer to the ss-cDNA 3′- OH extremities

Add and dissolve 25 µl of distilled water to the ss-cDNA adjusted and dried in step i) (in a Siliconized Eppendorf tube), centrifugate and collect the liquid at the bottom, process for 10 minutes in 65 °C, and quench. Centrifugate again for 1 second, add an additional 5 µl of X10TdT buffer (1.4 M cacodylic acid potassium (pH 7.6), 0.6 M-tris-base (19.3 g cacodylic acid (free acid) and 7.2 g trisma base (manufactured by Sigma Corp.) dissolved in 50 ml of re-distilled water, added to hydrated potassium powder, adjusted to pH 7.6, and bacteria reduced), 5 µl of chloride cobalt, 5 µl of DTT and 5 µl of dCTP, mix thoroughly, and centrifugate.

Add 5 µl of TdT (4 u/µl), mix lightly, and incubate for 10 minutes at 15 °C. Add 10 µl of 5M chloride sodium, 4 µl of 250 mMEDTA, and 36 µl of distilled water to this reactive solution, and treat under heat for 5 minutes at 70 °C.

Precipitate in ethanol after phenol extraction, and dry after rinsing.

iii) ds-cDNA creation

Add 26 µl of distilled water to the ss-cDNA samples (dried and stored in a siliconized Eppendorf tube) created in the aforementioned step ii) with dC-homopolymer added to the 3′ extremities, thoroughly dissolve the mixture, and quench after treating it for 5 minutes at 68 °C.

After centrifugating for 1 second, add 10 µl of X10cDNA buffer, 1 µl of DTT, and 10 µl of dATP, dGTP, dCTP, and dTTP, and 15 µl of oligo $(dG)_{12-18}$ and centrifugate after mixing. Next, add 13 µl of reverse transcriptase (5.8 u/µl) and store for 1 hour under 42 °C.

Add 20 µl of 5M chloride sodium, 8 µl of 250 mMEDTA, 2 µl of 20% SDS and 70 µl of distilled water to the reactive solution to stop the reaction.

Following standard methods, extract the phenol, precipitate the ethanol, and after rinsing and drying, add 40 µl of distilled water and dissolve the mixture, and after treating for 5 minutes at 62 °C, quench and centrifugate.

Add 10 µl of X10Klenow buffer (0.67MK-phosphoric acid buffer (pH 7.4), 67 mM chloride magnesium, 10 mMDTT), and 10 µl each of dATP, dGTP, dCTP, and

dTTP, mix well, add 10 μl of DNA polymerase1 (Klenow enzyme, 5 u/μl) and let react for 1 hour at 37 °C.

Add 40 μl of 5M chloride sodium, 16 μl of 250 mMEDTA, and 4 μl of 20%SDS and 140 μl of distilled water to stop the reaction, then carry out phenol extraction and ethanol precipitation using standard methods. If necessary, low molecular ds-cDNA can be removed at this stage using gel electrophoresis or neutral sucrose density slope methods.

Add and dissolve 100 μl of distilled water to the ds-cDNA, place the mixture on an ultrogel AcA44 column (gel bed height 28 cm) and fractionate approximately 0.6 ml at a time, gathering void volume fractions (measure the elution patterns using a liquid scintillation counter with Gerenkov methods).

Add 1/10 the amount of 3M acetate potassium and twice the amount of cold ethanol to this fraction, and after leaving the mixture for one hour at –70 °C, recover the developed ds-cDNA precipitate through centrifugation drying, rinse, and vacuum-dry.

iv) ds-cDNA Extremity Processing for Plasmid Insertion

Add and dissolve 37 μl of distilled water to the ds-cDNA adjusted in the aforementioned step iii), then add 5 μl of X10TdT buffer and 5 μl of dCTD, mix well, next add 3 μl of TdT, and incubate for 5 minutes at 37 °C.

After the reaction, add 10 μl of 5M chloride sodium, 4 μl of 250 mMEDTA and 36 μl of distilled water, and treat for 5 minutes at 70 °C, then extract phenol, precipitate ethanol, rinse, and vacuum dry.

v) Addition of PstI cut 3′ Extremity Oligo dG and Annealing with pBR322

Add 50 μl of X10 annealing buffer (0.1 M-tris-hydrochloric acid (pH 7.5), 1M chloride sodium, 10mMEDTA) to the ds-cDNA solution (450 μl) and mix thoroughly, and take 100 μl of that mixture and put it in an Eppendorf tube (1.5 ml, siliconized). Add 1 μl of pBR322 (oligo $(dG)_{10-20}$ tailed) and mix, treat for 5 minutes at 68 °C, then transfer to a constant-temperature water tank at 43 °C and incubate for 2 hours.

Turn off the incubator and allow to return to room temperature for at least 2 hours or more (overnight is acceptable), then store in a tube at 4 °C.

vi) Transformation

Using Dagert and Ehrlich methods, the conversion rate for the pBR322 DNA using RR1 as the host at $8.2 \times 10^4 - 1.4 - 10^7$ single transformation bulb/μg was attained.

(4) Create, as a probe, ^{32}P-labeled cDNA for the concentration fraction of the glycinin interim subunit mRNA from step (2).

(5) Using the probe created in step (4), sort the glycinin subunit clone from the cDNA library from step (3) using the colony hybridization method. Of the 1023 clones, 22 were positive.

(6) Of the 22 clones attained in the above step (5), clones with insertion portion lengths 1 kb or greater (16 clones) were selected and glycinin cDNA clones were identified using the "positive hybrid selection and in vitro translation" method. 1 of these clones had an insertion portion of approximately 2 kb. The northern plot hybridization results that used this clone as a probe showed the mRNA length to be approximately 2.2 kb, and the created cDNA was near a complete length.

The structure of one glycinin A_3 B_4 subunit cDNA among the cloned cDNA was as shown in Table 1.

Embodiment 2

The structure of one glycinin A_5 A_4 B_3 subunit cDNA structure attained using the same methods as Embodiment 2 was as shown in Table 2.

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Table 1
(Soybean Glycinin A_3B_4 cDNA Base Sequence)

[see source for sequence]

Table 2
(Soybean Glycinin $A_5A_4C_3$ cDNA Base Sequence)

[see source for sequence]